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Thymol enrichment from oregano essential oil by molecular distillation

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ABSTRACT

The objectives of this study were to obtain fractions enriched in thymol by molecular distillation of oregano essential oil and to evaluate their antioxidant properties. In addition, this operation was modelled considering the effects of evaporation temperature and feeding flow on thymol concentration in the residue fractions by means of artificial neural networks (ANN).

All residue fractions had a higher percentage of thymol than the distillates. The thymol composition in the residue fractions reached values up to 2.4-fold higher than in oregano essential oil. Higher concentrations of thymol produced lower values for IC_{50} according to DPPH· technique, indicating an increase in antioxidant properties.

During the storage test, all the residue fractions analysed showed better peroxide, conjugated dienes and conjugated trienes values than distillates and OEO when they were used in sunflower oil samples. The results of storage stability showed that residues and BHT are very good antioxidants.

The ANN created demonstrated good predictive ability for the operation of molecular distillation of oregano essential oil.

1. Introduction

In recent years, there has been a growing interest in the research of natural active products, such as essential oils and the extracts of some herbs and spices, for the development of alternative food additives for the prevention of growth of foodborne pathogens and lipid oxidation. This situation has motivated industry to search for separation systems that allow the concentration or purification of active compounds present in these renewable sources without significant damage.

Numerous studies on various essential oils from aromatic plants have reported that oregano essential oil has a significant antioxidant effect on lipid oxidation [4,6,26,24,11,18]. Many of these works attribute the antioxidant power to the phenolic monoterpenes carvacrol and thymol. Lagouri et al. [12] and Milos et al. [18] studied oregano essential oil rich in thymol and carvacrol and found a significant antioxidant effect on the lipid oxidation process. In addition, it was found that the essential oil had greater antioxidant activity than the individual active components, indicating a possible synergism between the constituents of the oil [25,17]. Other studies have confirmed that both compounds have antioxidant properties [27,18,11,15,17], and it has been suggested they may have synergism with minority oxygenated components [11].

Oregano is one of the most important commercial herb crops in Argentina. The major production areas of oregano and other herbs, spices and medicinal plants in Argentina are the central and southwestern regions, including the provinces of San Juan, Mendoza, Córdoba and San Luis [1]. Of the species commercially known as oregano, most of the production in Argentina comes from *Origanum vulgare* ssp. *vulgare*, *Origanum vulgare* ssp. *virens*, *O. x applii*, and *O. x majoricum* [6].

Substances from natural sources, like oregano essential oil, are usually heat sensitive and can be damaged if exposed to high temperatures. Molecular distillation is a high vacuum operation that causes a decrease in the boiling point of substances due to operating pressure reduction. This feature, combined with low residence time, allows the concentration or purification of compounds of interest with no deterioration of their natural properties. There is little information available about the application of molecular distillation for obtaining purified or enriched fractions from essential oils [3,16,5,10,22,20].

Phenomenological modelling of molecular distillation operations is very complex, and for this reason has been studied using new techniques, including Artificial Neural Networks (ANN) [21]. ANN have been used for modelling molecular distillation [21,23], but there is no information available in the literature about mod-

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elling oregano essential oil molecular distillation with this methodology.

The objectives of this study were to obtain fractions enriched in thymol from oregano essential oil by molecular distillation and to evaluate their antioxidant properties. In order to optimise the operating conditions of molecular distillation, this operation was modelled considering the effects of evaporation temperature and feeding flow on thymol concentration in the residue fractions by means of artificial neural networks.

2. Materials and methods

2.1. Materials

Oregano essential oil (OEO) was provided by PLATARIO SA, (Buenos Aires, Argentina). This essential oil was obtained by steam distillation of oregano (*Origanum vulgare* sp. *virens*), farmed in Barreal, Calingasta, province of San Juan, Argentina, located at 31° 40′ South latitude, 69° 29′ West longitude, 1650 m above sea level and harvested in March 2011. The essential oil was dried over anhydrous sodium sulfate, preserved in sealed flasks, and stored at 4–6 °C until the moment of analysis.

Refined sunflower oil, trademark "Natura" from AGD, General Deheza, Córdoba, Argentina, was used to storage assays.

2.2. Methods

2.2.1. Description of the process

Molecular distillation (MD) was performed in a DCC4 falling film distiller, manufactured by J.M. Pedroni and associated (Buenos Aires, Argentina). This equipment has an evaporation surface of 0.04 m^2 and an internal condensing surface of 0.02 m^2. The distiller is equipped with variable speed roller wipers. OEO was fed at room temperature and two fractions were obtained: distillate (D) and residue (R).

In this work, the study of thymol enrichment from OEO was performed using a central composite design with two factors and three replicates at central point. Preliminary works of this research group have shown that evaporation temperature and feed flow are the most relevant variables for the operation [22,14]; therefore they were selected as independent variables to evaluate the performance of OEO molecular distillation. Experiments were carried out in a randomized order to minimize the effect of unexpected variability in the observed response. The operative conditions of MD are presented in Table 1. OEO was introduced into the feed flask and degassed using a Venturi water pump to prevent bubbling and splashing during distillation under vacuum conditions. The condenser temperature was set at −2 °C and rotor speed was kept constant at 200 rpm. Evaporation temperature was varied between 23 and 37 °C and feed flow ranged from 0.3 to 1.7 ml/min. Evaporation pressure was fixed at $3 \cdot 10^{-3}$ bar.

Table 1
Operating conditions for molecular distillations.

Essay	F_{feed} (ml/min)	T_{evap} (°C)
1	1.5	25
2	1.5	35
3	0.5	25
4	0.5	35
5	0.3	30
6	1	37
7	1.7	30
8	1	23
9	1	30
10	1	30
11	1	30

Residue percentage (%*R*) is defined as the ratio between mass of residue fraction and mass of feed. Thymol concentration is calculated as the ratio between thymol mass in each residue or distillate and the total mass of that fraction, multiplied by 100, $\%T_R$ and $\%T_D$, respectively.

2.2.2. Chemical composition of oregano essential oil and of different fractions obtained by molecular distillation

The chemical composition of OEO and of different fractions obtained by MD was determined by gas chromatography–mass spectrometry, using a Perkin Elmer Clarus 600 equipment equipped with a flame ionization detector (FID). A Carbowax capillary column (60 m, 0.25 mm, 0.25 μm) was used. Oven temperature was held at 60 °C for 5 min and then it was gradually increased from 60 °C to 240 °C at 5 °C min^{-1}. Finally, it was kept constant at 240 °C for 10 min. Flow of carrier gas (N_2) was 1 ml/min. Injector and detector temperature were 250 and 350 °C, respectively. Samples were diluted in n-hexane (1/100 μl), and the injection volume was 1 μl. The components were identified by comparison of peak mass spectrum with the mass spectrum of pure standard substances. Main components where also identified by comparing their retention time with standard compounds. Relative concentrations were calculated according to peak area normalization given by TurboMass 5.4.2.

2.2.3. Antioxidant activity-DPPH· radical scavenging assay

Antioxidant capacity of the essential oil and the fractions obtained by MD was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH·) as stable radical, according to the methodology of Borgarello et al. [3]. To determine the free radical scavenging capacity, IC_{50} value was defined as the amount of sample, in mg/ml, that produces a decrease of the initial concentration of DPPH· to 50%. A high IC_{50} value indicates a weak free radical scavenging capacity, while a low IC_{50} value indicates a high free radical scavenging capacity.

2.2.4. Sampling and storage conditions

Samples of refined sunflower oil were enriched with OEO and with the residues and distillates obtained by MD that showed higher radical scavenging activity, i.e. R5, R6 and D5. Control sample of sunflower oil (SO) was also prepared under the same conditions without any antioxidant addition. At the same time, the natural antioxidants were compared with a synthetic antioxidant. BHT commercial antioxidant was added to a sunflower oil sample in a concentration of 0.02 % w/w, according to the maximum amount allowed in edible oils by Argentine Food Code [2]. OEO and different fractions were added at a concentration of 0.1 % w/w. This concentration was selected based on the results of earlier studies aimed at compromising between the antioxidant effect and sensory properties.

Samples were placed in open test tubes (exposed to air oxygen) and stored in the dark at 23 °C (room temperature). The environment was air-conditioned and temperature was monitored periodically. Samples of each product were removed from storage for chemical analyses at days: 0, 5, 15, 26, 46, 65 and 117. The identification of the samples is described below:

SO: Sunflower oil;
SOOEO: Sunflower oil enriched with natural OEO;
SOR5: Sunflower oil enriched with residue fraction of essay 5;
SOR6: Sunflower oil enriched with residue fraction of essay 6;
SOD5: Sunflower oil enriched with distillate fraction of essay 5;
SOBHT: Sunflower oil enriched with BHT commercial antioxidant.

2.2.5. Chemical analysis on samples from storage

Peroxide value (PV), conjugated dienes and trienes (CD and CT) were used to evaluate samples oxidation and to follow its evolution on time. PV was evaluated by the AOCS (1993) standard method and expressed as active oxygen miliequivalents per kilogram of oil ($meqO_2/kg$). CD and CT were measured in a spectrophotometer (UV–Vis Spectrophotometer Biotraza 752, Instrumental Pasteur, Buenos Aires, Argentina) at 232 nm and 268 nm, respectively. The results were reported as the sample extinction coefficient E (1%, 1 cm) (COI, 2001).

2.2.6. Molecular distillation modelling using artificial neural networks

The performance of molecular distillation in different operating conditions was modelled with an artificial neural network (ANN), using the neural network toolbox from Matlab 7.11.0 (R2010b). Evaporation temperature and feed flow were chosen as input variables of this process, while the response was thymol composition in the residue fraction. The structure chosen was a backpropagation network in which the number of neurons in the input and output layer correspond to the number of input and output variables respectively.

Backpropagation neural network requires real entries included in the range [−1 1]. For this reason, input data was codified using the following Eq. (1):

$$\begin{aligned} Fcod &= 1.4215 \cdot F - 1.4264 \\ Tcod &= 0.1429 \cdot T - 4.2857 \end{aligned} \quad (1)$$

After choosing the architecture of the network, it must follow a training stage where experimental data is used to make the network imitate the operation behaviour. Once it is trained, there is a validation phase where experimental data is compared with network approximation values. The training stage is possible due to the learning ability of the ANN. Network learning is a process in which some parameters of the ANN are fixed through a continuous stimulation in the environment where the system is located.

The results achieved by the ANN model were confronted with the experimental data obtained. For this purpose, Relative Error (2) was defined as the difference between the experimental data and the results obtained by the ANN model. Correlation coefficient (R-value) (3) of the linear model relating the outputs of the ANN model and the targets (experimental data) was used to choose the best model.

$$\text{Relative Error} = \frac{|y_{exp} - y_{ANN}|}{y_{exp}} \quad (2)$$

$$R\text{-value} = \sqrt{\frac{\sum_{i=1}^{n}(y_{ANN_i} - \hat{y})^2}{\sum_{i=1}^{n}(y_{est_i} - \hat{y})^2}} \quad (3)$$

where y_{exp} is the experimental data; y_{ANN} is the result obtained by the ANN model $\hat{y}$ is the experimental media value defined as (4).

$$\hat{y} = \sum_{i=1}^{n} y_{exp_i} \quad (4)$$

2.2.7. Statistical analyses

Analytical determinations results were the average of triplicate measurements from three independent samples. The data was analysed using InfoStat software, version 2012.p (Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Córdoba, Argentina). Statistical differences were estimated by ANOVA test at 95% level ($p < 0.05$) of significance. Whenever ANOVA indicated a significant difference, a pair-wise comparison of means by least significant difference (LSD) was carried out. Regression equations were used to determine the effect of the independent variable (storage time) on chemical parameters (PV, CD and CT). The regression analysis was performed adjusting a simple lineal model: $y = \beta_0 + \beta_1 \cdot x$, where '$y$' was the dependent variable (PV, CD and CT); and 'x' was the independent variable (time).

3. Results and discussion

Oregano essential oil contains 18.51% of thymol. Other major substances found in the raw material were *trans*-sabinene hydrate (34.67%); γ-terpinene (10.99%); cyclopropane, 1-cyclopropylethy nyl-2-methoxy-3,3-dimethyl (5.71%) and oct-1-en-3-dyl acetate (4.14%).

These results agree with those obtained by Asensio et al. [1] and Dambolena et al. [6], who also found large percentages of *trans*-sabinene hydrate, thymol and γ-terpinene in oregano essential oils from Argentina. *Trans*-sabinene hydrate is usually present at low percentages or it is not found in essential oils from elsewhere [8,4,13,9,7].

The compounds identified in OEO and in most relevant fractions are presented in Table 2.

Other experimental results of molecular distillation, in particular the residue percentage (%R) and thymol concentration in all the residue and distillate fractions (%T_R and %T_D) are presented in Table 3. The operating conditions used allowed the obtainment of a broad range of %R. Assay 1 presented the highest % R and the lowest %T_R and %T_D, respectively. Assay 5 had the lowest %R and the highest %T_R and %T_D, respectively.

All residues had a higher percentage of thymol than the distillates. This is because most of the substances that compose oregano essential oil presented higher volatility than thymol; heavier compounds remain in the residue.

Higher concentrations of thymol were achieved when most of the feeding was distilled, that is, when low percentages of residue were obtained. The operative conditions that resulted in low %R were low F_{feed} and high T_{evap}. All distillates presented lower thymol percentages than OEO.

The maximum enrichment was achieved in R6, in which the thymol composition was 2.4-fold higher than in oregano essential oil. Thymol recovery in R6, calculated as the ratio between mass of thymol in the residue stream and mass of thymol in the feed stream multiplied by 100, was 72.20%.

Table 2
Chemical composition of OEO, D5, R5 and R6 analysed by GC–MS.

Compound	OEO	D 5	R 5	R 6
α-Pinene	1.00	0.95	0.28	0.16
β-Phellandrene	3.38	3.75	0.88	0.51
β-Myrcene	1.13	1.55	0.27	0.15
α-Terpinene	0.91	1.25	0.21	0.00
(+-)-Limonene	0.80	1.11	0.22	0.13
(+)-Sabinene	0.67	1.05	0.00	0.00
(e)-Ocimene	2.07	3.09	0.22	0.14
γ-Terpinene	10.99	15.15	3.43	0.00
o-Cymene	1.44	1.75	0.47	0.34
oct-1-en-3-yl acetate	4.14	4.65	1.87	1.53
trans-Sabinene hydrate	34.67	35.94	27.20	27.02
δ-Carene	2.67	2.61	2.70	3.01
β-Cariophyllene	2.60	2.74	2.07	2.74
Cyclopropane, 1-cyclopropyl ethynyl-2-methoxy-3,3-dimethyl	5.71	6.49	3.60	3.52
3-Carene	0.44	0.38	0.44	0.48
α-Terpineol	3.20	2.95	3.96	4.17
β-Bisabolene	0.78	0.54	1.62	1.91
trans Piperitol	0.32	0.24	0.55	0.57
(-)-β-Caryophyllene epoxide	1.01	0.18	4.05	3.85
Spathulenol	0.65	0.00	2.79	2.70
Thymol	18.52	10.30	42.04	44.45

Table 3
Experimental results of molecular distillation under different operating conditions. Condenser temperature: −2 °C. $P = 3 \cdot 10^{-3}$ bar. OEO IC_{50}: 1.5 mg/ml. %T OEO: 18.51%.

Essay	%R	$\%T_R$	$\%T_D$
1	69.18	24.98	1.25
2	58.8	30.39	2.39
3	60.5	29.29	2.76
4	27.6	39.88	9.14
5	22.78	42.04	10.30
6	30.08	44.45	7.87
7	64.92	30.94	3.40
8	61.58	32.00	4.34
9	52.19	30.83	3.49
10	45.81	34.51	6.21
11	52.21	30.33	5.50

% *R*: residue percentage.
% thymol R: thymol concentration at the residue fraction.
% thymol D: thymol concentration at the distillate fraction.

3.1. Free radical scavenging activity

The relationship between radical scavenging capacity in the residues (IC_{50}R) and distillates (IC_{50}D) as a function of thymol composition, $\%T_R$ and $\%T_D$ respectively, is presented in Fig. 1. The residue fractions obtained by molecular distillation showed an increase in radical scavenging capacity, while the distillates showed a decrease in that antioxidant property.

Higher concentrations of thymol produce lower values for IC_{50}R, indicating an increase in antioxidant properties. The relationship between the variables is nonlinear and the dispersion observed may be caused by the presence of minor oxygenated compounds and by synergisms and antagonisms among certain components and thymol.

The distillate fractions presented the highest values for IC_{50} and low percentages of thymol.

3.2. Oxidative stability test

Sunflower oil contained 0.65 mg vitamin E/ml and no synthetic antioxidants. Its initial peroxide value was 1.63 mEq O_2/kg oil.

The changes in the values for peroxide, conjugated dienes and conjugated trienes of the SO, SOR5, SOR6, SOD5, SOOE and SOBHT samples during storage at 23 °C are shown in Fig. 2. In general, these variables increased during the storage time in all products.

SO and SOBHT had the highest and the lowest PV, respectively, during storage. At day 46, SOR6, SOR5 and SOBHT did not show any significant differences among them, but they were significantly different with respect to SOOEO and SOD5. At day 117 all of the samples exhibited significant differences from each other, and the order of increase of peroxide value was: SOBHT < SOR5 < SOR6 < SOOEO < SOD5 < SO.

Conjugated dienes presented the same behaviour as PV. The order of increase of CD values was SOBHT < SOR5 < SOR6 < SOOEO < SOD5 < SO. SO had the highest CD and CT values during storage. At day 117 all of the samples showed significant differences from each other and the increasing order of CT was the same as that observed for CD values.

The residues analysed had lower PV, CD and CT values than oregano essential oil and its distillates. The results for storage stability showed that the residues and BHT are very good antioxidants.

Olmedo et al. [19] studied the oxidative stability of fried–salted peanuts with the addition of essential oils. Laurel essential oil, oregano essential oil and BHT showed similar antioxidant activity and increased the shelf life of fried-salted peanuts.

Regression equations for PV evolution for each product (SO, SOOEO, SOR5, SOR6, SOD5, SOBHT) at 23 °C are presented in Table 4. In all cases, the equations have positive slopes indicating an increase with storage time. All regressions have R^2 higher than 0.97, indicating that they are a good predictive tool. Therefore, those equations could be used to predict the effect of storage time at 23 °C on sunflower oil.

According to these equations, SO and SOD5 had higher rates of increment for PV than the other samples, indicating than D5 is a fraction with poor antioxidant properties. SOR6, SOR5 and SOBHT had lower slopes, meaning a higher oxidative stability. According to Argentine Food Code, 10 meqO_2/kg is the maximum level of peroxide value allowed for edible oils [2]. This value may be useful as an endpoint of quality for sunflower oils enriched with different fractions. The time required to reach the peroxide value of 10 meqO_2/kg was obtained from the linear regression of peroxide value–time curves for different samples. Using these equations, PV values of 10 meqO_2/kg were reached after 26 days in SO, 35 days in SOD5, 37 days in SOOEO, 43 days in SOR6, 46 days in SOBHT and 48 days in SOR5. The maximum level of peroxide value allowed by Argentine Code is reached in few days because the samples were exposed to air oxygen.

These results are in agreement with those obtained by the DPPH· technique, strengthening the idea that fractions of OEO enriched in thymol have good antioxidant properties, indicating that residue fractions enriched in antioxidants provide protection against lipid oxidation in sunflower oils.

3.3. Artificial neural network model

The backpropagation neural network structure chosen is formed by an input layer with two neurons, one for each operating variable studied, a hidden layer with an hyperbolic tangent sigmoid transfer function (tansig) containing three neurons and an output layer with linear transfer function. Higher amounts of neurons increased the deviation of the values predicted by the model with respect to the experimental results. The R^2 of the linear regression between the objectives and outputs of the network

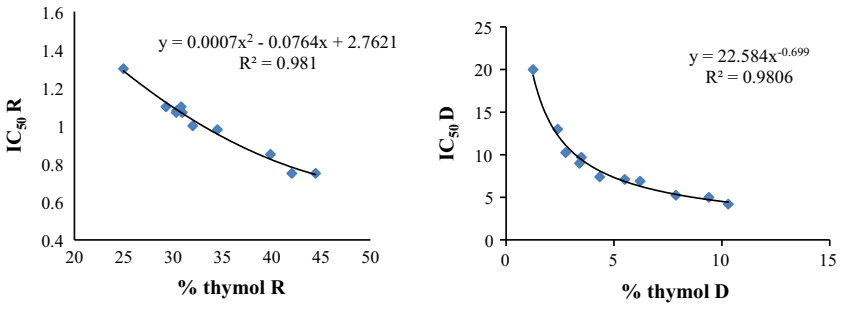

Fig. 1. Variation of IC_{50} with thymol composition in residue and distillate fractions.

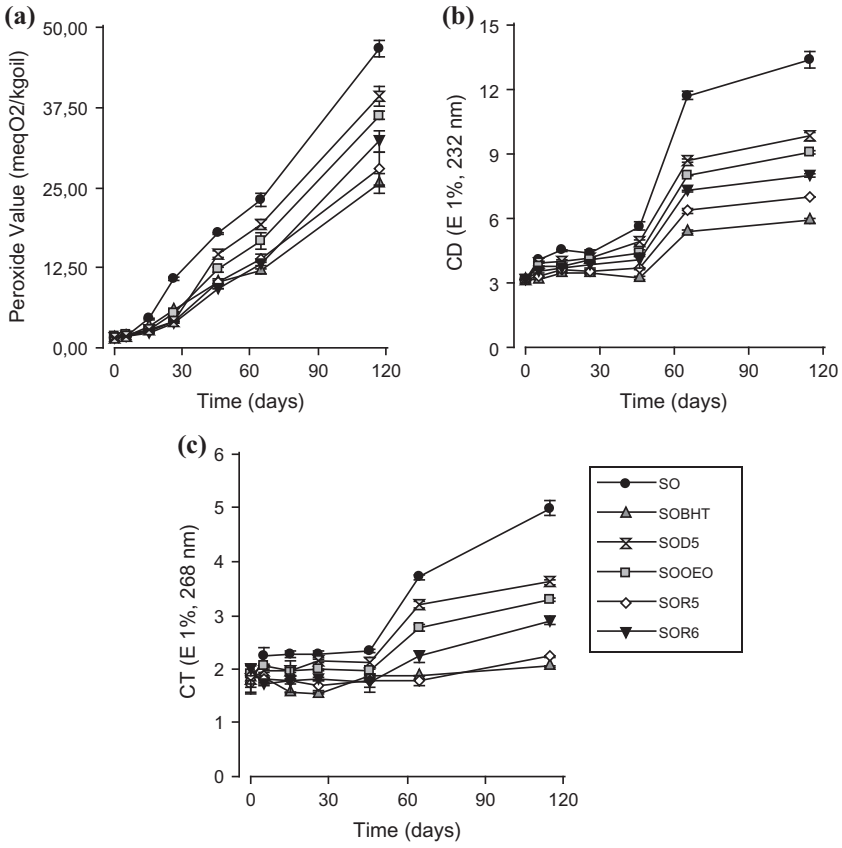

Fig. 2. (a) Peroxide values (PV), (b) Conjugated dienes (CD) and (c) Conjugated trienes (CT) in sunflower oil (SO), sunflower oil enriched with OEO (SOOEO), sunflower oil enriched with residue 5 (SOR5), sunflower oil enriched with residue 6 (SOR6), sunflower oil enriched with distillate 5 (SOD5) and sunflower oil enriched with commercial antioxidant BHT (SOBHT) during the storage time at 23 °C.

Table 4
Regression equation and R^2 for peroxide value (PV) during storage time.

Dependent variable	Sample	Lineal regression coefficients		
		β_0	β_1	R^2
PV	SO	−0.33	0.39	0.99
	SOD5	−1.70	0.34	0.98
	SOOEO	−1.10	0.30	0.97
	SOR6	−1.65	0.27	0.97
	SOR5	−0.53	0.24	0.98
	SOBHT	0.44	0.21	0.98

Regression coefficients from the general regression equation: $y = \beta_0 + \beta_1 \cdot x$, where y = dependent variable (PV) and x = independent variable (days of storage).

Table 5
Experimental results, ANN outputs and deviation between these values (in absolute value) for thymol composition in the residue fractions.

Experimental data	ANN output	Deviation
0.2498	0.2523	1.00
0.3039	0.3101	2.04
0.2929	0.2840	3.04
0.3988	0.4023	0.88
0.4204	0.3496	16.84
0.4445	0.4494	1.10
0.3094	0.3008	2.78
0.3200	0.3054	4.56
0.3083	0.3094	0.36
0.3451	0.3094	10.34
0.3033	0.3094	2.01

was 0.84. The experimental data, network outputs and the deviation between these values are presented in Table 5.

The simulation of the ANN created for thymol composition in the residue fraction as a function of evaporation temperature and feed flow is shown in Fig. 3. The neural network was capable of modelling the chosen variable.

An intermediate zone was observed, where changes in operative conditions did not produce important changes in thymol composition.

For conditions where little evaporation was achieved, thymol in the residue fraction was not considerably concentrated because a

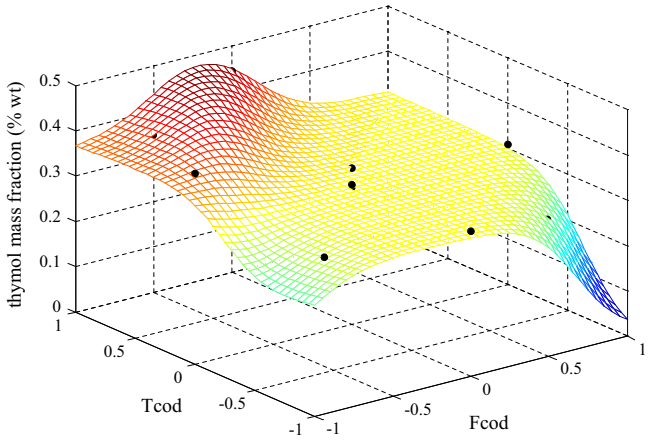

Fig. 3. Simulation of the ANN model for thymol composition in the residue fraction and experimental data (•) as a function of evaporation temperature and feed flow.

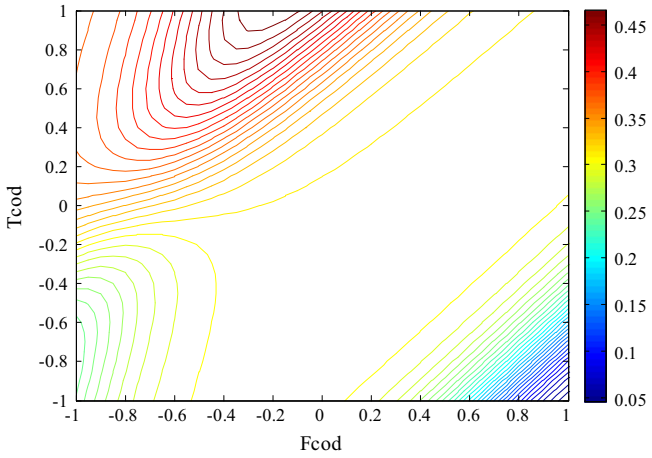

Fig. 4. Contour curves of the ANN model for thymol composition in the residue fraction.

large amount of compounds still remained in the mixture, diluting it.

Low feed flows and high temperatures produced the highest thymol enrichment in the residues, as it can be seen in the contour curves shown in Fig. 4.

4. Conclusions

The results of the present work indicate that molecular distillation is a suitable operation for obtaining enriched fractions from oregano essential oil. Furthermore, the addition of these thymol-enriched fractions to sunflower oil improved its stability by preventing lipid oxidation. All of the residue fractions had higher percentages of thymol than the distillates, and also presented higher antioxidant activity according to the DPPH· technique and to the storage stability test.

The residue fractions could be used as natural antioxidants in other similar food products with high lipid contents increasing their shelf life, improving their stability and preventing the loss of sensory and nutritional quality.

The ANN created demonstrated good predictive ability for the operation of molecular distillation of oregano essential oil. It could be used to ensure a defined product quality, as a first step towards the design of a process or a system of automatic process control in real time.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.seppur.2015.08.035.

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